Characterization of the phase I metabolism of resibufogenin and evaluation of the metabolic effects on its antitumor activity and toxicity

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Abbreviations:
ABT, 1-aminobenzotriazole; CYP3cide, PF-4981517; DAD, diode array detector; ESI, electrospray ionization; HLF, human embryonic lung fibroblasts; HLM, human liver microsomes; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; MLM, ICR/CD-1 mouse liver microsomes; DLM, beagle dog liver microsomes; RLM, SD rat liver microsomes; GLM, Dunkin-Hartley guinea pig liver microsomes; CyLM, cynomolgus monkey liver microsomes; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; NSCLC, non-small cell lung cancer; 5-HRB, 5β-hydroxylresibufogenin; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; CYP, cytochrome P450; PBS, phosphate buffered saline; RB, resibufogenin; rhCYP450, recombinant human cytochrome P450; TCM, Traditional Chinese medicine; TEPA, triethylenethiophosphoramide; UFLC, Ultra-Fast liquid chromatography.
ABSTRACT

Resibufogenin (RB), one of the major active compounds of traditional Chinese medicine Chansu, has displayed great potential as a chemotherapeutic agent in oncology. However, it is a digoxin-like compound, and also exhibits extremely cardiotoxic effects. The present study aims to characterize the metabolic behaviors of RB in humans as well as to evaluate the metabolic effects on its bioactivity and toxicity. The Phase I metabolic profile in human liver microsomes was characterized systemically, and the major metabolite was identified as marinobufagenin (5β-hydroxyresibufogenin, 5-HRB) by using LC-MS and NMR techniques. Both cytochrome P450 (CYP) reaction-phenotyping and inhibition assays using CYP-selective chemical inhibitors, demonstrated that CYP3A4 was mainly involved in RB 5β-hydroxylation with much higher selectivity than CYP3A5. Kinetic characterization demonstrated that RB 5β-hydroxylation in both HLM and human recombinant CYP3A4 obeyed the biphasic kinetics, displaying similar apparent kinetic parameters. Furthermore, 5-HRB could significantly induce cell growth inhibition and apoptosis in A549 and H1299 by facilitating apoptosome assembly and caspase activation. Meanwhile, 5-HRB displayed very weak cytotoxicity of human embryonic lung fibroblasts, and a greater tolerance to acute toxicity in mice. In summary, CYP3A4 dominantly mediated 5β-hydroxylation and was found as a major metabolic pathway of RB in the human liver, while its major metabolite (5-HRB) displayed better druglikeness than its parent compound RB. Our findings lay a solid foundation for RB metabolism studies in humans, and encourage further research on the bioactive metabolite of RB.
INTRODUCTION

Chansu, the dried skin secretions of the giant toad (containing *Bufo bufo gargarizans* Cantor and *Bufo melanostictus* Schneider), is an important Traditional Chinese medicine (TCM) and is widely used in China and other Asian countries, for treating a number of ailments including swelling, pain, heart failure and cancer in the clinic (Nogawa et al., 2001; Li et al., 2010). It is also used as a raw medicinal material in many famous complicated formulas of TCMs as over-the counter drugs, namely Liushen Pill, Shexiang Baoxin Pill and Kyushin (Shimizu and Morishita, 1996; Ma et al., 2009). Resibufogenin (RB) is one of the major active compounds in Chansu with a content of 4%-10%, and it is regarded as a vital marked compound for the quality control of Chansu and its related TCM preparations (Ma et al., 2009). Many studies in recent years indicate that it possesses significantly pharmacological and toxicological effects including cardiotonic, anesthetic, anti-tumor and cardiotoxic effects. Furthermore, both preclinical and clinical studies show that RB and its analogues possessing the skeletons of bufadienolide are potent anti-cancer agents (Mijatovic et al., 2007; Newman et al., 2008). It has been reported that RB exhibits strong cytotoxic activities against human myeloid leukemia, prostate cancer and human hepatoma cells with IC$_{50}$ values of approximately 10-50 nM (Kamano et al., 1998). The cytotoxic activities of RB against human hepatoma Bel-7402, human gastric cancer BGC-823 and human cervical carcinoma HeLa were comparable to or stronger than taxol (Ye et al., 2005). With the intensive investigation of the underlying molecular mechanisms, it evidenced that RB and its analogue-mediated Na$^+$, K$^+$-ATPase targeting could circumvent various chemoresistance pathways as a novel way of attacking resistant cancer cells. Moreover, the various molecular pathways that the Na-K targets can be more deleterious to biologically aggressive cancer cells than to normal cells (Mijatovic and
Kiss, 2013). This collective information demonstrated that RB is a chemotherapeutic candidate that holds a great potential to serve as a chemotherapeutic agent in oncology (Kamano et al., 1998; Qi et al., 2011).

However, these digoxin-like bufadienolides are cardiotoxic (Brubacher et al., 1999; Gowda et al., 2003; Kostakis and Byard, 2009) and have been shown to be associated with a high mortality rate (Xie et al., 2001). It is well-known that the metabolic clearance of a given drug and the formation of new drug metabolites can significantly influence the efficacy and toxicity in vivo (Guengerich, 2006), but there is still limited attention given to the metabolic effects of RB in humans. Recently, the metabolic characteristic of RB in rats was illustrated by isolation and identification of metabolites excreted in bile (Zhu et al., 2013). Consistent with the metabolic fate of cinobufagin (a RB analogue) (Ning et al., 2010; Ma et al., 2011), it is suggested that 3-O-epimerization of RB is a rapid and important metabolic reaction in rats (Chen et al., 2012). However, the metabolic behaviors of RB in humans as well as the metabolic effects on its bioactivities and toxicity have not been well characterized.

Therefore, the objectives of this study are 1) to elucidate the phase I metabolic pathway(s) and kinetic characterization of RB in human liver microsomes, 2) to identify the main CYP enzymes responsible for phase I metabolism of RB, 3) to reveal the metabolic effects on its bioactivity and toxicity using in vitro and in vivo methods.
MATERIALS AND METHODS

Reagents

Resibufogenin (Fig. 1) was isolated from Chansu by one of the authors (J.N.) and unambiguously identified by NMR and MS techniques. Its purity was above 98% as determined by ultra-Fast liquid chromatography-DAD (UFLC-DAD). 1-aminobenzotriazole (ABT), clomethiazole, D-glucose-6-phosphate, furafylline, glucose-6-phosphate dehydrogenase, PF-4981517 (CYP3cide), 8-methoxypsoralen, NADP⁺, omeprazole, quinidine and sulfaphenazole were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ketoconazole was obtained from ICN Biomedicals Inc. (Aurora, Ohio, USA). Montelukast was from Beijing Aleznova Pharmaceutical (Beijing, China). Triethylenethiophosphoramide (TEPA) was purchased from Acros Organics (Geel, Belgium). All other reagents were either of LC grade or the highest grade commercially available.

25 donors mixed pooled human liver microsomes (HLM), the human individual microsomal samples as well as liver microsomes of male ICR/CD-1 mouse, male Sprague-Dawley rat, male Dunkin-Hartley guinea pig, male Beagle dog, and male Cynomolgus monkey were purchased from Research Institute for Liver Diseases (RILD, Shanghai, China). cDNA-expressed recombinant human cytochrome P450 isoforms (rhCYP) including rhCYP1A2, rhCYP2A6, rhCYP2B6, rhCYP2C8, rhCYP2C9, rhCYP2C19, rhCYP2D6, rhCYP2E1, rhCYP3A4, rhCYP3A5 and rhCYP3A7, which were derived from baculovirus-infected insect cells co-expressing NADPH-CYP reductase, were from CYPex (Dundee, UK). All microsomal samples and rhCYP isoforms were stored at -80 °C until use. Human NSCLC A549 and H1299 cell lines and the embryonic human lung fibroblasts (HLF) cell line were obtained from ATCC (Manassas, VA). The cleaved caspase-9 antibody was
purchased from Cell Signaling Technology (Danvers, MA, USA), GAPDH was from ProteinTech (Chicago, IL, USA), and cytochrome c was purchased from Santa Cruz Biochemicals (Santa Cruz, CA).

Animals

All animals were maintained and used in accordance with the animal protocol approved by the local research ethics review board of the Animal Ethics Committee of Dalian Medical University, and all experimental procedures described were carried out in accordance with the Declaration of Helsinki. ICR/CD-1 mice weighing between 18 and 21 g were obtained from the laboratory animal center of Dalian Medical University. The mice were fasted overnight with free access to water before the date of the experiment.

In vitro metabolism of resibufogenin

Incubation Conditions. The incubation mixture, with a total volume of 200 µL, consisted of 100 mM potassium phosphate buffer (pH 7.4), NADPH-generating system (1 mM NADP⁺, 10 mM glucose-6-phosphate, 1 unit/mL of glucose-6-phosphate dehydrogenase, 4 mM MgCl₂), and liver microsomes. In all experiments, RB (25 mM previously dissolved in acetonitrile) was serially diluted to the required concentrations and the final concentration of acetonitrile did not exceed 1% (v/v) in the mixture. After pre-incubation at 37 ºC for 3 min, the reaction was initiated by adding NADPH-generating system and further incubated at 37 ºC in a shaking water bath. The reaction was terminated by the addition of ice-cold acetonitrile (100 µL). The mixture was kept on ice until it was centrifuged at 20,000 g for 10 min at 4 ºC. Aliquots of supernatants were stored at -20 ºC until analysis. Control incubations without NADPH-generating system or without substrate or without microsomes were carried out to ensure that metabolite formation was microsome- and NADPH-dependent. All
incubations throughout the study were carried out in three experiments performed in duplicate with S.D. values generally below 10%.

**Assays with recombinant human cytochrome P450 isoforms.** Eleven cDNA-expressed human cytochrome P450 isoforms co-expressing NADPH-P450 reductase (rhCYP1A2, rhCYP2A6, rhCYP2B6, rhCYP2C8, rhCYP2C9, rhCYP2C19, rhCYP2D6, rhCYP2E1, rhCYP3A4, rhCYP3A5 and rhCYP3A7) were used to screen the involved isoform(s) for the hydroxylation of RB in HLM. The incubations were carried out under the standard assay procedure as described in the text. To generate adequate metabolites for detection, a relatively high substrate concentration (25 and 250 µM) was selected and incubated with each of the recombinant CYPs (40-160 nM) at 37 °C for 30 min. UFLC equipped with DAD detector was used to quantify the metabolite(s) of RB.

**Chemical Inhibition assays.** The hydroxylation of RB in HLM with the absence or presence of selective inhibitors for different CYP isoforms was measured to explore the involved enzyme(s) for this metabolism. In brief, RB (10 µM) was incubated in HLM (0.05 mg protein/ml) with an NADPH-generating system in the absence (control) or presence of known CYP isoform-specific inhibitors. The selective inhibitors and their concentrations were as follows (Bjornsson et al. 2003): montelukast (2 µM) for CYP2C8 (Walsky et al. 2005), sulfaphenazole (10 µM) for CYP2C9, omeprazole (20 µM) for CYP2C19, quinidine (10 µM) for CYP2D6, clomethiazole (50 µM) for CYP2E1, ketoconazole (1 µM) for CYP3A. Inhibition by furafylline (10 µM) for CYP1A2, 8-methoxypsoralen (2.5 µM) for CYP2A6, TEPA (50 µM) for CYP2B6 (Rae et al. 2002), CYP3cide (2 µM) for CYP3A4 (Walsky et al., 2012) and ABT (500 µM) for broad CYPs (Emoto et al. 2003) were examined by adding RB after pre-incubation with NADPH-generating system at 37 °C for 40 min.
Kinetic Characterization. To estimate kinetic parameters of RB hydroxylation in human liver microsomes as well as recombinant CYP3A4, the incubation conditions were optimized to ensure that formation rates of 5-HRB were in relation to incubation time and protein concentration in the linear range. RB (1, 2.5, 5, 10, 25, 50, 75, 100, 150, 200 and 250 µM) was incubated with HLM (0.05 mg protein/mL) or incubated with rhCYP3A4 (5 nM) for 20 min. All incubations were carried out in three independent experiments in duplicate. The apparent $K_m$ and $V_{max}$ values were calculated from nonlinear regression analysis of experimental data according to the Michaelis-Menten equation, and the results were graphically represented by Eadie-Hofstee plots. Kinetic constants were reported as the value ± standard error (S.E.) of the parameters estimate.

Correlation studies. The formation rates of the metabolites described for RB (5 µM, near $K_m$ value) were determined in a panel of HLM prepared from 12 individual donors. RB was incubated with HLM (0.05 mg protein/mL) for 15 to 60 min to ensure that the formation rate of 5-HRB were in relation to incubation time and protein concentration in the linear range. These values were compared to the levels of CYP3A4 or CYP3A5 in 12 individual HLM. The concentrations of CYP3A4 and CYP3A5 in HLM were determined by liquid chromatography-tandem mass spectrometer, using multiple reaction monitoring mode and isotope labeled peptide as the internal standards. Specific peptides of EVTNFLR (for CYP3A4) and SLGPVGFMK (for CYP3A5) were selected for their quantification by using transition ions of m/z 439.7/549.3, and m/z 468.3/678.5, respectively. The correlation parameter was expressed by the linear regression coefficient (r). P < 0.05 was considered statistically significant.

UFLC-DAD and UFLC-ESI-MS Analysis. The UFLC system was equipped with a CBM-20A
communications bus module, an SIL-20ACHT autosampler, two LC-20AD pumps, a
DGU-20A3 vacuum degasser, a CTO-20AC column oven and an SPD-M 20A diode array
detector. A Shim-pack XR-ODS (750 mm × 2.0 mm, 2.2 µm, Shimadzu) analytical column
with an ODS guard column (5 mm × 2.0 mm, 2.2 µm, Shimadzu) was used to separate RB
and its metabolites. The mobile phase consisted of CH₃CN (A) and water containing 0.2%
(v/v) formic acid (B), with the following gradient profile: 0–2 min, 96%–76% B; 2-5 min,
76%–64% B; 5–10 min, 64%–49% B; 10-13 min, 49%–5% B; and 13-16 min, balanced to
96% B. The flow rate was 0.4 mL/min and the column temperature was kept at 40 ºC. RB and
its metabolites were detected at 299 nm and quantified according to the calibration curves of
authentic standards.

Shimadzu LC-MS-2010EV (Kyoto, Japan) instrument with an ESI interface was used for
identification of RB and its metabolite(s). Mass detection was performed in both positive-ion
mode (ESI⁺) and negative ion mode (ESI⁻) from m/z 100 to 800. The detector voltage was set
at +1.75 kV, and −1.55 kV for positive and negative ion detection, respectively. The curved
desolvation line temperature (CDL) and the block heater temperature were both set at 250 ºC.
Other MS detection conditions were as follows: interface voltage, 4 kV; CDL voltage, 40 V;
nebulizing gas (N₂) flow was 1.5 L/min and the drying gas (N₂) pressure was set at 0.06 MPa.
Data processing was performed using the LC-MS Solution version 3.41 software.

Metabolite purification and identification.

In vitro biosynthesis and the isolation of major metabolite. The predominant metabolite was
biosynthesized by using the mixture of CyLM (90%) and HLM (10%). Above all, the major
biotransformed product by CyLM was characterized by UFLC-DAD-ESI-MS and it was
found to have retention time, UV spectra, and mass spectra identical to the metabolite in
HLM. The incubation system was scaled up to 250 ml. RB (200 μM) was incubated with the liver microsomes (final protein concentration, 1.0 mg/mL) and the NADPH-generating system (1 mM NADP⁺, 10 mM glucose-6-phosphate, 1 unit/ml of glucose-6-phosphate dehydrogenase, and 4 mM MgCl₂) for 4 h at 37 °C. Under these conditions, approximately 35% of RB was converted to 5-HRB. Acetonitrile (250 mL) was added to the reaction mixture to precipitate the protein. After centrifuged at 20,000 × g for 15 min at 4 °C, the supernatant was separated and extracted with ethyl acetate (250 mL × 3). The organic layer was combined and dried in vacuo, and the residue was re-dissolved in acetonitrile (1.5 mL) and injected into HPLC instrument. The HPLC system (SHIMADZU, Kyoto, Japan) consisted of a SCL-10A system controller, two LC-10AT pumps, a SIL-10A auto injector, a SPD-10AVP UV detector and a C18 column (4.6 mm × 150 mm, 5 μ) was used to separate RB and its metabolite. The mobile phase was 65% methanol in water. The eluent was monitored at 299 nm with a flow rate of 1.2 mL/min, and the fractions containing 5-HRB were collected and dried in vacuo. The purity of 5β-hydroxy-resibufogenin was above 98% by HPLC-UV analysis.

NMR Spectroscopy. All NMR experiments were performed on a Varian INOVA-500 NMR spectrometer. ¹H and ¹³C NMR spectra (at 500 and 125 MHz, respectively) were measured at room temperature (22 °C). Chemical shifts are given on the δ scale and were referenced to tetramethylsilane at δ = 0 ppm for ¹H and ¹³C spectra.

Cytotoxicity Assays of resibufogenin and its metabolite

Cell culture. Human NSCLC A549 and H1299 cell lines and HLF cell line were maintained in either RPMI 1640 medium or DMEM medium supplemented with 10% fetal bovine serum. All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂.
Cell viability assay. Cell viability was determined by a MTT assay (Roche Diagnosis, Indianapolis, IN). Briefly, lung cancer cell lines (6×10^3 cells/well) were seeded into 96-well plates. Cells were allowed to adhere overnight, and then the cells were changed to fresh medium containing various concentrations of 5-HRB or RB dissolved in DMSO (final concentration, 0.1%). After 48 h incubation, the growth of cells was measured. The effect on cell viability was assessed as the percent cell viability compared with untreated control group, which were arbitrarily assigned 100% viability. The compound concentrations required to cause 50% cell growth inhibition (IC_{50}) were determined by interpolation from dose-response curves. Experiments were performed by triplicate and at least three independent experiments were carried out.

In vitro migration assay. A scratch assay (wound-healing assay) was performed to detect cell migration. The cells were grown to full confluence in six-well plates and wounded with a sterile 100 μL pipette tip after 6 h of serum starvation and then washed to remove detached cells from the plates. Cells were treated with indicated doses of 5-HRB in full medium and kept in a CO₂ incubator. After 48 h, medium was replaced with phosphate buffered saline (PBS) buffer, the wound gap was observed, and cells were photographed using a Leica DM 14000B microscope fitted with digital camera.

Colony formation assay. To analyze the cell sensitivity to 5-HRB, we used a colony formation assay in vitro. Briefly, A549 cells (1.0×10^3 per well) were seeded in six-well plate containing 2 ml growth medium with 10% FBS and cultured for 24 h. Then, removed the medium, and cells were exposed to various concentrations of 5-HRB. After 18 h, cells were washed with PBS and supplemented fresh medium containing 10% FBS. The cultures were maintained in a 37 °C, 5% CO₂ incubator for 14 days, allowing viable cells to grow into
macroscopic colonies. Removed the medium, and the colonies were counted after staining with 0.1% crystal violet.

Confocal immunofluorescence. For confocal microscopy analysis, cells grown on chamber slides were washed in PBS and fixed for 30 min at room temperature with 4% paraformaldehyde. And then the cells were permeabilized with 0.2% TritonX-100 for 3 min. The samples were blocked with 10% bovine serum albumin (BSA) in PBS for 30 min. Antibodies against cytochrome c in the 1% blocking solution were added to the sample and incubated for overnight at 4°C. Non-immune rabbit IgG and mouse IgG were included as controls. Following five 10-min washes with PBS, fluorescein isothiocyanate- and rhodamine-conjugated secondary antibodies were added in blocking solutions and incubated for 1 h in darkroom at room temperature. After five additional 10-min washes, samples were examined with a Leica confocal microscope, and images were processed with Image-Pro Plus 5.1 software. More than 100 cells were inspected per experiment, and cells with typical morphology were presented.

Western blot analysis. Cell lysate proteins were separated by electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide minigels and then electrophoretically transferred to a PVDF membrane. Western blots were probed with the specific cleaved caspase-9 antibody. GAPDH was used as controls for sample loading. The protein bands were detected by enhanced chemiluminescence. Similar experiments were performed at least three times. The total protein concentration was determined using a BCA protein assay kit.

Acute toxicity assay. Acute toxicities of RB and 5-HRB were tested using a variation of the method described by Litchfield and Wilcoxon (1949). Six different doses of each drug were used for the LD₅₀ index determination, with each experimental group being composed of five
mice for this purpose. The compounds RB and 5-HRB were administered by intraperitoneal injection. The animals were observed at 10 min, 30 min and 60 min after administration to access the numbers of deaths and possible toxicological symptoms.

**Data analysis and statistics**

Kinetic constants for RB hydroxylation by HLM or rhCYP3A4 were obtained by fitting experimental data to the biphasic Michaelis-Menten kinetics using Origin (OriginLab Corporation, Northampton, MA). The biphasic Michaelis-Menten equation is $v = \frac{(V_{\text{max}1} + [S])}{(K_{\text{m}1} + [S])} + \frac{(V_{\text{max}2} + [S])}{(K_{\text{m}2} + [S])}$, where $v$ is the rate of reaction, $V_{\text{max}}$ is the maximum velocity, $K_{\text{m}}$ is the Michaelis constant, and $[S]$ is the substrate concentration.

The IC$_{50}$, representing the inhibitor concentration that inhibits 50% of control activity, is determined by nonlinear curve fitting with Origin. Kinetic constants and IC$_{50}$ values are reported as the value ± S.E. of the parameter estimate.
RESULTS

Biotransformation of Resibufogenin by Human Liver Microsomes.

The in vitro metabolic profiles of RB were characterized after incubation with the human hepatic microsomes (0.3 mg protein/mL) along with the NADPH-generating system for 30 min (Fig. 2). The metabolites were characterized by UFLC-DAD-ESI-MS. The positive-ion mode was adopted for LC-MS analysis, due to the fact that it is more sensitive than the negative-ion mode for analysis of target analytes. Mass spectra were dominated by intact molecular ions [M+H]+ and significant acetonitrile adducts [M+CH3CN+H]+. The m/z for the [M+H]+ and [M+CH3CN+H]+ of the metabolite in human hepatic microsomes were 401.2 and 442.2, respectively. Accordingly, the molecular weight (MW) of metabolite was calculated to be 400 (increasing m/z 16 compared with RB), indicating this metabolite was a mono-hydroxylated form.

Identification of the main Mono-hydroxylated Metabolite.

In order to elucidate the main metabolic site of RB, this metabolite was purified and further identified by 1H-NMR and 13C-NMR. The spectral data of the mono-hydroxylated metabolite was listed in Table 1. Compared with NMR data of RB, the 13C-NMR spectrum of this metabolite showed the disappearance of tertiary carbon at δ 35.5 (C-5), meanwhile the additional oxygenated quaternary carbon at δ 74.3 (CH) was observed. The carbon signals of C-4, C-6 and C-10 shifted down to δ 36.8 (Δδ+3.8), δ 34.2 (Δδ+8.6), δ 40.6 (Δδ+5.6), respectively. While the upfield shifts of C-1 (Δδ-4.4) and C-19 (Δδ-6.8) were observed, due to the γ-gauche effect, all of which suggesting an additional hydroxyl group was located at C-5 position. Furthermore, the NMR data of metabolite agreed well with the spectral data of marinobufagenin reported previously (Ma et al., 2008). On the basis of above analysis, the
hydroxylated metabolite was identified as 5β-hydroxyresibufogenin (5-HRB, Fig. 1).

**Chemical Inhibition Assays.**

A broad specificity CYP inactivator and nine selective inhibitors of major CYP isoforms were used to screen the CYP isoform(s) responsible for the formation of 5-HRB in HLM (Fig. 3A). ABT, a broad specificity CYP inactivator, completely inhibited the formation of 5-HRB, suggesting that CYPs were responsible for RB hydroxylation in HLM. Among the selective inhibitors tested, ketoconazole (CYP3A inhibitor) strongly inhibited the catalytic activity of HLM, further implying the prominent role of CYP3A in RB hydroxylation. Furthermore, CYP3cide, a potent and specific time-dependent inhibitor of CYP3A4 (Walsky et al., 2012), exhibited similar inhibitory effects on RB 5β-hydroxylation in HLM and rhCYP3A4 (Supplementary data 1). Meanwhile, inhibitors of other CYP isoforms did not exhibit a significant inhibition (less than 20% inhibition, p > 0.05) towards the formation of 5-HRB. These findings suggest that the formation of 5-HRB was selectively catalyzed by CYP3A4.

**Assays by Recombinant Human CYP Isoforms.**

In order to further verify the CYP isoform(s) involved in RB metabolism in humans, the formation of 5-HRB was determined using eleven rhCYP isoforms. After incubation at 37 °C for 30 min, one hydroxylated metabolite was formed exclusively by CYP3A family (including CYP3A4, CYP3A5 and CYP3A7), no metabolite was observed in the incubation with rhCYP1A2, rhCYP2A6, rhCYP2B6, rhCYP2C8, rhCYP2C9, rhCYP2C19, rhCYP2D6 and rhCYP2E1 (less than 0.01 nmol/min/nmol P450) (Fig. 3B). The formation rates of rhCYP3A4 for 5-HRB were 16.4 ± 0.7 and 53.8 ± 3.6 nmol/min/nmol P450 at substrate concentrations of 25 and 250 μM, respectively. However, RB 5β-hydroxylation rates of rhCYP3A5 and rhCYP3A7 were very limited, with less than 100-fold than 5-HRB formation.
rate catalyzed by rhCYP3A4, at the substrate concentration of 25 μM.

**Kinetic Characterization.**

Over the whole concentration range tested, RB 5β-hydroxylation in HLM and rhCYP3A4, obeyed the biphasic kinetics, as evidenced by Eadie-Hofstee plot (Fig. 4). Additionally, the kinetic characterization of RB 5β-hydroxylation in HLM and rhCYP3A4 displayed the similar apparent kinetic parameters. In HLM, $K_{m1}$ and $K_{m2}$ values for 5-HRB formation were 1.64 and 36.5 μM, respectively, while the $V_{max1}$ and $V_{max2}$ values for formation of 5-HRB were 0.66 and 2.58 nmol/min/mg protein, respectively. Similarly, the $K_{m1}$ and $K_{m2}$ values for 5-HRB formation in rhCYP3A4 were 3.25 and 93.6 μM, respectively, and the $V_{max1}$ and $V_{max2}$ values were 18.3 and 42.6 nmol/min/nmol P450, respectively (Table 2).

**Correlation studies.**

CYP3A4 and CYP3A5 are the most abundant CYP3A enzymes in the adult human livers, and they share 83% identity in amino acid sequence. Although CYP3A5 has generally been considered less important than CYP3A4, there lie many controversial issues in the contribution of CYP3A5 to the total clearance of CYP3A, which may be caused by the polymorphism and expression in interindividual and interracial variability (Daly, 2006). Therefore, in the present study, the correlations of CYP3A4 or CYP3A5 expression levels with RB hydroxylation were investigated to reveal which one of these two CYPs is the predominant enzyme that was responsible for metabolism of RB in the human livers. The formation rates of 5-HRB were determined in a panel of HLMs prepared from 12 individual donors. These values were compared with the protein levels of CYP3A4 or CYP3A5 in 12 individual HLMs. As shown in Fig. 5, the linear regression coefficient (r) for 5-HRB formation rate and CYP3A4 concentration was 0.857 (P < 0.001), and for 5-HRB formation
rate and CYP3A5 concentration was 0.083 (P = 0.799). There was a high correlation between 5-HRB formation rate and CYP3A4 expression levels, in contrast, a poor correlation of 5-HRB formation rate with CYP3A5 protein levels, which fully indicates the prominent contribution of CYP3A4 towards RB hydroxylation in HLM.

**RB and its metabolite both inhibited NSCLC cell growth and changed cell morphology.**

To evaluate the hydroxylation effect on cytotoxicity of RB, we firstly determined the effect of 5-HRB against cell proliferation of human lung cancer cells by MTT assay. As shown in Fig. 6, both 5-HRB and RB resulted in dose-dependent growth inhibition of NSCLC cells, and they could markedly reduce cell-to-cell contact and induce cell shrinkage compared with the control groups. The IC$_{50}$ values for cell viability inhibition of 5-HRB and RB were 91.5 ± 7.8 nM, 23.6 ± 5.4 nM in A549, and 718.1 ± 19.6 nM, 262.5 ± 16.7 nM in H1299, respectively. However, the IC$_{50}$ values for cell viability inhibition in HLF were approximately 1000-fold than those in NSCLC cells, which fully suggesting that 5-HRB displayed the highly selective cytotoxicity against tumor cells.

**5-HRB suppressed cell morphology change and the clonal formation.**

The changes of cell morphology mediated by treatments of 5-HRB were detected in A549 cells. We analyzed the inhibition of clonal formation ability in A549 cells treated with 5-HRB at the appropriate concentrations. As shown in Fig. 7A, 5-HRB highly inhibited colony formation and resulted in a remarkable decrease at colony formation ratio. Further, we analyzed the inhibition of 5-HRB on cell motility in A549 cells by employing wound-healing assay. Treatment with 5-HRB inhibited cell migration compared with the control group without any agent treatment. The wounding space between cell layers was occupied mostly by the migrating cells at 48 h after making a scratch in control cells. By contrast, the gap of
cells was not occupied by the migrating cells treated with 5-HRB at the indicated doses (Fig. 7B). These results suggest that 5-HRB has the perfect properties in inhibiting cell colony formation and migration for NSCLC cells.

**5-HRB induced apoptosis through regulating cytochrome-c/caspase signaling.**

Treatment with 5-HRB at the doses of 20 µM and 100 µM induced 26% and 50% apoptotic cells in A549, respectively (Fig. 8A). To determine the effect of 5-HRB on apoptosis, we detected the expression of pro-apoptotic proteins caspase-9 in A549 cells by Western blot analysis. 5-HRB markedly increased the expression levels of cleaved caspase-9, compared with the control group (Fig. 8C). We next performed immunofluorescence imaging (IF) analysis to monitor changes in the subcellular localization of cytochrome-c in A549 cells to determine whether 5-HRB could induce cytochrome-c release. As shown in Fig. 8B, treatment with 5-HRB (50 nM or 100 nM) markedly triggered the release of cytochrome-c from the inter-mitochondrial space into the cytosol (Fig. 8B). These results demonstrate that 5-HRB induced cell growth inhibition is associated with the increase of apoptosis which may ascribe to the facilitation of the downstream cytochrome-c-dependent apoptosome assembly and caspase activation in the cytosol.

**Acute toxicity and cardiotoxicity evaluation.**

It is well known that mice and rats are less sensitive to bufadienolides than are dogs or humans. However, we kept to use mice in the present study (for the obvious reason of compound availability and the observed similar metabolic profile of RB, see Supplementary data 2, 3 and 4) for investigating *in vivo* safety of RB and its hydroxylated metabolite (5-HRB). After the intraperitoneal administration of RB (4, 5, 7.1, 8.5 and 10 mg/kg) and of 5-HRB (23, 27, 32.5 and 38 mg/kg), the death numbers were recorded. And at the dose of 10
mg/kg for RB, all animals were death and showed hypokinesia, deepening breath, heart rate decrease and orthotonus. Similarly, after the intravenous administration of RB (0.5, 1, 1.8, and 3 mg/kg) and of 5-HRB (3, 5.5, 8 and 12 mg/kg), the death numbers were recorded. The intraperitoneal and intravenous LD50 for RB is 6.22 and 2.40 mg/kg, respectively. However, for 5-HRB, the major metabolite of RB by HLM, its intraperitoneal and intravenous LD50 value is 30.6 and 9.71 mg/kg, which preliminarily suggest that 5-HRB has the less toxicity than that of RB.

Additionally, the primary cardiotoxicities of RB and 5-HRB in neonatal rat primary cardiac myocytes were also evaluated in present study. RB and 5-HRB did not influence the cell morphology significantly, but the beat frequencies were decreased with increasing concentrations of RB and 5-HRB. The LDH release rate of cardiac myocytes treated by RB was higher than that of 5-HRB (Supplementary data 7), which suggesting that 5-HRB exhibited less cardiotoxicity than did RB.
DISCUSSION

Recent advances in the investigation of bufadienolides have indicated that RB is a type of biologically active molecule with a wide variety of physiological and pharmacological functions (Mijatovic et al., 2007; Newman et al., 2008). The previous study reported that the cytotoxic and cardiotoxic action of bufadienolides were varied greatly and the substituted groups of steroid skeletons could significantly influence to the above effects. It leads us to query whether the biotransformation of RB which results in either active or inactive metabolites, could make a significant influence on its efficacy and toxicity. To answer the question, we studied the metabolism profile of RB in HLMs; then firstly identify the main metabolite and the involved metabolic enzyme. It was disclosed that 5-HRB was the main metabolite in HLM, which rapidly and specifically catalyzed by CYP3A4. Kinetic characterization demonstrated that RB 5β-hydroxylation in both HLM and recombination CYP3A4 obeyed the biphasic kinetics, with high affinity Km and a large in vitro intrinsic clearance value. The metabolic features indicated that the formation of 5-HRB may significantly influence the in vivo anticancer effects of RB. In addition, compared with other bufadienolides in Chansu, the intrinsic clearance of RB in rhCYP3A4 (5.63 μL/min/pmol P450), was higher than those of cinobufagin (2.61 μL/min/pmol P450) and bufalin (1.70 μL/min/pmol P450), respectively (Ge et al., 2013). It implies that tiny changes in the substituted chemical groups of steroid skeletons (such as 16-OAC or 14β-OH groups) would significantly influence the binding and catalysis activities between CYP3A4 and bufadienolides. These findings may provide the reference information on the further investigation of the interaction as well as the metabolism-structure relationship between CYP3A4 and other bufadienolides.
Previous studies have been revealed that RB can be rapidly metabolized in rats (with a short half-life of 7.5 min, i.v.), while 3-O-epimerization is the most important metabolic pathway of RB in rats (Zhu et al., 2013). The in vitro incubation study showed that 3-epi-resibufogenin is a dominate metabolite of RB (100 µM) in rat liver microsomes with a NADPH-generating system. In sharp contrast, 5-HRB is the major metabolite in liver microsomes from mouse, dog, guinea pig, monkey and human (Supplementary data 2). These findings agree well with our previous metabolic investigation of cinobufagin, which is a structural analogue of RB (Ma et al., 2007; Ning et al., 2010; Ma et al., 2011). The significant differences in metabolic behaviors between rat and human suggested that rat could not serve as a preferred surrogate model for in vivo study of RB, and the in vivo pharmacological or toxicological data obtained in rat models previously may be the misleading. For example, it has been reported that RB has the capability to antagonize marinobufagenin (5-HRB)-caused hypertension, proteinuria, intrauterine growth restriction and weight gain, in a rat model of human preeclampsia (Vu et al., 2006, Horvat et al., 2010; Puschett et al., 2010; Puschett et al., 2012), but these bioactivities have not been observed in other animals. Our results make it conceivable that marinobufagenin will be generated when RB is administrated in human, while 3-epi-RB and its derivatives are generated in rat and these metabolites may serve as antagonists of marinobufagenin. Therefore, it is necessary to reevaluate those in vivo data of RB and its analogues obtained in rat, by using a suitable surrogate model for simulating their metabolism in humans.

It is particularly noted that marinobufagenin (5-HRB), a major metabolite of RB in HLM, is one of the endogenous mammalian bufadienolides (Fedorova et al., 2001; Prassas and Diamandis, 2008; Uddin et al., 2011; Uddin et al., 2012). As an endogenous steroid,
marinobufagenin participates in a variety of physiological and pathophysiological processes including the regulation of Na\(^+\)-K\(^+\)-ATPase activity and the pathogenesis of arterial hypertension in mammals including humans (Bagrov et al., 1998; Bagrov et al., 2005). It has been reported that the plasma levels of marinobufagenin are closely associated with proteinuria and preeclampsia in pregnancy (Puschett et al., 2010; Puschett et al., 2012). Taking into account that marinobufagenin is the major metabolite of RB in HLMs, the administration of RB could increase the plasma concentration of marinobufagenin. It is easily conceivable that the administration of RB will make the condition more serious in pregnancies with preeclampsia, due to the production of extra marinobufagenin by CYP3A4 mediated-hydroxylation of RB. Therefore, adequate consideration should be taken on the prescription of TCMs containing RB or marinobufagenin.

Recent investigations demonstrate that the Na\(^+\)-K\(^+\)-ATPase alpha subunit is a promising new target for developing anticancer agents (Mijatovic et al., 2007; Mijatovic et al., 2008). As a natural inhibitor of Na\(^+\)-K\(^+\)-ATPase, the study on antineoplastic activity of RB and its metabolite is even more noteworthy. In the present study, the cytotoxic activities of 5-HRB and RB were evaluated in NSCLC, and 5-HRB still exhibited the excellent cytotoxic activities against NSCLC including A549 and H1299 cells. By monitoring the apoptosis associated protein, it indicated the anti-cancer bioactivities of 5-HRB were partially mediated through the activation of cytochrome c/caspase-dependent apoptotic signaling pathways for A549 cells. However, the attentions should be paid to the discrepancy between the present findings and the reported structure-cytotoxic activity relationship of bufadienolides which demonstrated that 5-hydroxylation reduced the anticancer activity significantly (Kamano et al., 1998; Ma et al., 2011). It reflected that the metabolic effect on bufadienolides
may change case by case and make it meaningful to investigate the biotransformation of bufadienolides which holds a great potential to serve as a chemotherapeutic agent in oncology.

Considering the approximately 1000-fold discrepancy between the IC₅₀ values for cell viability inhibition in NSCLC and HLF cells, it suggested that malignant cells are more susceptible to the effects of RB and 5-HRB than are normal cells. To further clarify and compare the safety of RB and 5-HRB, we evaluated the acute toxicity of RB and 5-HRB in vivo. Firstly, a comparative study of RB hydroxylation in humans and four common experimental animals (including monkey, dog, guinea pig and mouse) was performed to determine a suitable animal model for the toxicity evaluation (Supplementary data 2-5). It suggested that 5β-hydroxylation of RB is the major pathway in four animal species, and mouse is selected as a preferred model due to its similar metabolic profiles, enzyme and catalytic efficacy to those of human. Our results indicated that 5-HRB possesses a relatively high tolerability in vivo.

From the views of drug metabolism and pharmacological evaluation, the CYP3A-mediated RB 5β-hydroxylation in humans generates a bioactive and more polar metabolite marinobufagenin, implying that RB serves as a prodrug and its in vivo anticancer effects can be conserved after biotransformation. Additionally, we also found that marinobufagenin displays superior metabolic stability compared with RB, the further biotransformation of marinobufagenin in HLM is very slow (data not shown). These findings make us believe that marinobufagenin has a good druglikeness and can be considered as a potential leading anti-cancer compound. Taken together, marinobufagenin can be considered as a promising antitumor agent, due to its excellent cancer therapeutics, metabolic stability, and the
relatively high tolerability in the human body.

In summary, the profile of phase I metabolism of RB was firstly characterized. The major metabolite of RB in HLM was identified as 5β-hydroxyresibufogenin (5-HRB) and CYP3A4 was the major isoform responsible for RB 5β-hydroxylation with high selectivity. In addition, a significant species difference in 5β-hydroxylation was elucidated: in sharp contrast to the important involved 3-epimerization of RB in rat liver microsomes, 5β-hydroxylation plays the dominant role in the metabolism of RB in humans. Furthermore, the major metabolite of RB could significantly induce cell growth inhibition and apoptosis in A549 and H1299 by facilitating apoptosome assembly and caspase activation, all of which implies that 5-HRB may play an important role for the antitumor activities of RB in human. Our findings lay a solid foundation for further studies on RB metabolism in humans, and our in vitro and in vivo safety revaluation regarding to the bioactive metabolite of RB, encourage further researches on the bioactive metabolite of RB.
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Authorship Contributions

Participated in research design: Ma, Ge, and Yang

Conducted experiments: Ning, Yu, Hu, Huo, Wu

Contributed new reagents or analytic tools: Wang, and Deng

Performed data analysis: Ning, Yu, and Hou

Wrote or contributed to the writing of the manuscript: Ning, Ge, and Ma
REFERENCE


Nogawa T, Kamano Y, Yamashita A and Pettit GR (2001) Isolation and structure of five new
cancer cell growth inhibitory bufadienolides from the Chinese traditional drug Ch’an Su. *J Nat Prod* **64:**1148-1152.


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Ning and Yu contributed equally to this work.

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LEGENDS FOR FIGURES

Figure 1. The 5β-hydroxylation of RB in HLM.

Figure 2. Representative UFLC profile of RB and its metabolite in HLM. RB (100 µM) was incubated in human liver microsomes (0.3 mg/mL) with NADPH-generating system at 37 °C for 30 min.

Figure 3. Assignment of isozyme(s) involved in RB hydroxylation. (A) Inhibition of RB 5β-hydroxylation by selective CYP450 inhibitors in HLM. (B) The formation of metabolite by rhCYP450. Results are expressed as the mean ± S.D. from three experiments in duplicate.

Figure 4. Michaelis-Menten plots of RB metabolism in HLM (A) and rhCYP3A4 (B). An Eadie-Hofstee plot is shown as an inset to illustrate biphasic kinetics. Data points represent the mean of triplicate determinations.

Figure 5. The correlation between the CYP3A4 or 3A5 protein levels and RB hydroxylation rates. The correlation parameter was expressed by the linear regression coefficient (r). P < 0.05 was considered statistically significant.

Figure 6. RB and 5-HRB inhibited cell viability and changed morphology. (A) Human lung cancer A549, H1299 cells and human embryo lung fibroblast (HLF) cells were treated with RB or 5-HRB under normal culture medium at the indicated doses. (B) The changes in cell morphology and spreading in A549 cells treated with RB or 5-HRB for 48h were observed and cells were photographed using a microscope fitted with the digital camera.

Figure 7. 5-HRB suppressed cell colony formation and migration. Human A549 cells were treated with 5-HRB at the indicated doses for appropriate time. (A) The tumor cell A549-induced colony formation was analyzed, and the colony formation rate was calculated. (B) Cell migration was analyzed by a wound-healing assay. A549 cells were seeded in 6-well
plates and grown to full confluence. The migration rate was calculated and presented as the mean ± SD of three separate experiments. (*P<0.05, **P<0.01, significant differences between 5-HRB treatment groups and DMSO vehicle control groups).

Figure 8. 5-HRB induced apoptosis by modulating cytochrome c/caspase signaling. Human A549 cells were treated with 5-HRB at the indicated doses. At 24h after treatment, the apoptosis was determined by a FACS analysis (A); the release of Cytc in A549 cells was determined by immunofluorescence imaging analysis to monitor Cytc release from the mitochondrial intermembrane space into the cytosol (B) and the levels of the cleaved caspase-9 protein in A549 cells was analyzed by Western blot (C). The apoptosis is represented by relative percentages of apoptotic cells versus that in DMSO-treated cells. (*P<0.05, **P<0.01, significant differences between 5-HRB treatment groups and DMSO vehicle control groups).
Table 1. $^1$H-NMR (500 MHz, DMSO) and $^{13}$C-NMR (125 MHz, DMSO) spectral data for 5-HRB.

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Table 2. Kinetic parameters of RB 5β-hydroxylation determined in HLM and rhCYP3A4

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<th>Enzyme source</th>
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<th>$K_{m1}$</th>
<th>$V_{m2}$</th>
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<td>HLM</td>
<td>0.663 ± 0.138</td>
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<td>CYP3A4</td>
<td>18.3 ± 1.3</td>
<td>3.25 ± 0.40</td>
<td>42.6 ± 0.8</td>
<td>93.6 ± 10.2</td>
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$K_m$ values are micromolar concentrations, $V_{max}$ values are nanomoles per minute per milligram for liver microsomes or nanomole per minute per nanomole of P450 for CYP3A4. The range of substrate concentrations was 1 to 250 μM. Each value was the mean ± S.D. of three determinations performed in duplicate.
Fig. 1

Resbufagenin (RB) \[\xrightarrow{\text{CYP3A4}}\] 5β-Hydroxy-resbufagenin (5-HRB)
Fig. 3

A

% Control activity remaining

N.D. 100 80 60 40 20 0

ABT (CYPs) Furaayline (1A2) Triclopidine (2B6) Montelukast (2C9) Sulfaphenazole (2C9) Omeprazole (2C19) Quinidine (2D6) Chlomethiazole (2E1) Ketoconazoloe (3A) CYP3Ccal (3A4)

B

5-HRB formation rate (nmol/min/nmol P450)

N.D. N.D. N.D. N.D. N.D. N.D. N.D. N.D.

1A2 2A6 2B6 2C8 2C9 2C19 2D6 2E1 3A4 3A5 3A7

25 μM 250 μM
Fig. 5

A. $r = 0.857$ (P < 0.001)

B. $r = 0.083$ (P = 0.799)